

Secreted proteins and genes in fetal and neonatal pig adipose tissue and stromal-vascular cells¹

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ABSTRACT: Although microarray and proteomic studies have indicated the expression of unique and unexpected genes and their products in human and rodent adipose tissue, similar studies of meat animal adipose tissue have not been reported. Thus, total RNA was isolated from stromal-vascular (S-V) cell cultures (n = 4; 2 arrays; 2 cultures/array) from 90-d (79% of gestation) fetuses and adipose tissue from 105-d (92% of gestation) fetuses (n = 2) and neonatal (5-d-old) pigs (n = 2). Duplicate adipose tissue microarrays (n = 4) represented RNA samples from a pig and a fetus. Dye-labeled cDNA probes were hybridized to custom microarrays (70-mer oligonucleotides) representing more than 600 pig genes involved in growth and reproduction. Microarray studies showed significant expression of 40 genes encoding for known adipose tissue secreted proteins in fetal S-V cell cultures and adipose tissue. Expression of 10 genes encoding secreted proteins not

known to be expressed by adipose tissue was also observed in neonatal adipose tissue and fetal S-V cell cultures. Additionally, the agouti gene was detected by reverse transcription-PCR in pig S-V cultures and adipose tissue. Proteomic analysis of adipose tissue and fetal and young pig S-V cell culture-conditioned media identified multiple secreted proteins including heparin-like epidermal growth factor-like growth factor and several apolipoproteins. Another adipose tissue secreted protein, plasminogen activator inhibitor-1, was identified by ELISA in S-V cell culture media. A group of 20 adipose tissue secreted proteins were detected or identified using the gene microarray and the proteomic and protein assay approaches including apolipoprotein-A1, apolipoprotein-E, relaxin, brain-derived neurotrophic factor, and IGF binding protein-5. These studies demonstrate, for the first time, the expression of several major secreted proteins in pig adipose tissue that may influence local and central metabolism and growth.

Key words: adipose tissue, cell culture, fat cell, gene microarray, proteomics, secreted factor

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J. Anim. Sci. 2006. 84:1666–1681
doi:10.2527/jas.2005-539

INTRODUCTION

Adipose tissue secretes a wide variety of proteins that include, among others, leptin, adiponectin, and IL-6 (reviews, Trayhurn and Wood, 2004; Gimeno and Klamman, 2005; Hauner, 2005). Levels of mRNA for 9 factors secreted by adipose tissue were differentially regulated by food deprivation in the rat (Bertile and Raclot, 2004).

Gene expression in rat, mouse, and human adipose tissue has been studied in a global manner with cDNA microarrays (Gabrielsson et al., 2000; Nadler et al., 2000; Soukas et al., 2000; 2001; Castro-Chavez et al., 2003). A global approach allows for the evaluation of adipose tissue endocrine factors in the context of other secreted factors. In meat animal studies the expression of a number of adipose tissue genes including genes encoding several secreted factors has been examined, but a global approach has not been employed (review, Hausman and Hausman, 2004). We report, herein, the results of the first gene microarray studies of neonatal and fetal pig adipose tissue and fetal pig stromal vascular (S-V) cell cultures.

Using conventional approaches we, and others, have studied many adipose tissue secreted proteins including several paracrine/autocrine factors (review, Hausman et al., 2001). However, it is critical to supplement conventional approaches with a proteomics approach in

¹Mention of a trade name, proprietary product, or specific equipment does not constitute a guarantee or warranty by the USDA and does not imply its approval to the exclusion of other products that may be suitable. The authors gratefully acknowledge David Crandall and Amy Hreha, Wyeth Research Labs, Philadelphia, PA, for their time and effort and expertise in conducting all the plasminogen activator inhibitor-I assays in these studies.

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Received September 22, 2005.

Accepted February 1, 2006.

studies of adipose tissue secreted proteins because proteomics approaches are global and not inherently limited as are conventional techniques. Several proteomic studies of adipose tissue have been published (Lanne et al., 2001; Sanchez et al., 2001; Corton et al., 2004), but there are no published proteomic studies of meat animal adipose tissue. Therefore, we used several currently available proteomic approaches to evaluate proteins secreted by neonatal and fetal porcine adipocytes and adipose tissue on a more global basis. The studies presented herein represent an important first step in our efforts to identify the role of adipose tissue secreted factors in regulating puberty and growth in pigs.

MATERIALS AND METHODS

Gene Expression Analysis

The protocols used in these experiments were approved by the USDA, Richard Russell Center, Agricultural Research Service Animal Care and Use Committee. From the University of Georgia swine herd, 5- to 7-d-old postnatal pigs (C42 \times 280, PIC, Franklin, KY) and fetal pigs obtained by surgical removal at the designated times were used.

Pig cDNA Microarrays. Custom cDNA microarrays (Telechem International Inc., Sunnyvale, CA) were prepared by spotting 70-mer oligonucleotides that were designed from 560 pig gene sequences (brain, ovary, pituitary, and adipose tissues). Oligonucleotides were produced from expressed sequence tags from the Meat Animal Research Center, ARS, USDA genetic libraries that had at least 90% homology to known genes in The Institute of Genome Research pig gene index. Sequence-optimized 70-mers minimize cross-hybridization and differentiate between homologous genes, while allowing an equal representation of each gene.

Arrays were triple-spotted for each gene and contained buffer alone and lambda DNA as negative controls and *Escherichia coli* genes at different concentrations as positive controls. Total RNA was purified (Qiagen Inc., Valencia, CA) and reverse transcribed to DNA probes. Probes were labeled with Cy 3 and Cy 5 dyes and hybridized to custom microarrays (Telechem International Inc.). The hybridization step was standardized over a period of time with control experiments that demonstrated reproducible results. The hybridization solution included GlassHyb (Clontech, Mountain View, CA) with 10 μ g of salmon sperm DNA, and 35 pmol of Cy3 and Cy5 targets. Hybridization at 47°C for 12 h was followed by a 10-min low stringency wash at 45°C in 1 \times SSC, 0.2% SDS, and then a 10 min wash in 0.1 \times SSC, 0.2% SDS with a final wash of 4 min in 0.1 \times SSC.

The slides were scanned with a GSI Lumonics ScanArray 5000 (GSI Lumonics Inc., Billerica, MA), and image analysis was done with ArrayVision version 6.0 software. Fluorescent intensities were corrected for background, followed by normalization among arrays with a version of a global mean normalization procedure

that allows comparison of data across multiple microarray slides. Global normalization based on mean intensities was performed as follows: 5% of the distant outliers were discarded from the calculation of the mean (5% trimmean) for each microarray based on local background subtracted signal intensities; the average of the 5% trimmeans was calculated across all microarrays. The 5% trimmean for each microarray was divided by the average of the 5% trimmeans (across all microarrays) to produce a normalization factor for each microarray. To produce normalized intensities, the intensity of every gene for each microarray was divided by the normalization factor for that microarray. Background fluorescent intensity determination was based on a local background method. Areas were chosen surrounding each spot and used to compute a number that represented the background for that spot.

Adipose Tissue S-V Cell Cultures from 90-d Fetal Pigs for Microarray Analysis. Cultures of adipose tissue S-V cells from 90-d-old fetuses were prepared conventionally (Hausman, 2003) and treated with 10 μ M of troglitazone (Biomol Inc., Plymouth Meeting, PA) + fetal bovine serum (FBS, Sigma Aldrich, St. Louis, MO) from d 0 to 3 of culture, followed by insulin, transferrin, and sodium selenite (ITS, Sigma Aldrich) from d 3 to 6. Control cultures received FBS alone from d 0 to 3, followed by ITS from d 3 to 6. Cultures were harvested for total RNA on d 6. The RNA from only 1 fetal age, 90 d, was used because of the prohibitive cost of microarrays and microarray processing. Ninety-day-old fetuses were selected because this age represents the midway point of fetal pig adipocyte/adipose tissue development based on in vitro and in vivo studies (Hausman and Hausman, 1993).

Microarray Analysis of Neonatal Adipose Tissue and Fetal S-V Cells. The RNA was purified from subcutaneous adipose tissue from 105-d-old fetuses (n = 2, 2 arrays) and young pigs (n = 2, 2 arrays) and 90-d fetal S-V cell culture homogenates (n = 4; 2 arrays, 2 cultures/array). A total of 4 microarrays was used in the fetal and young pig adipose tissue study, and a total of 2 microarrays was used in the fetal S-V culture study. Each of the 4 adipose tissue arrays represented RNA from a fetus or a pig with duplicate arrays/fetus or pig.

Normalized fluorescent intensities were averaged in young pig and fetal adipose tissue arrays, and the means were compared by Student's *t*-tests. Normalized fluorescent intensities were also averaged across age in adipose tissue arrays and across treatments in fetal S-V cell culture arrays for the determination of expression of genes overall. Signal:noise ratio (S:N) was used to evaluate the reliability of the signal (Yang et al., 2003). The noise was the SD of the background density. The S:N of negative controls for the adipose tissue arrays was 0.9 ± 0.5 . Genes with $S:N \geq 4$ were considered positive signals to ensure distinction from background with a >99% statistical significance (Yang et al., 2003).

Agouti RNA Isolation and Reverse Transcription-PCR Analysis. Total RNA was isolated from subcuta-

neous adipose tissue from neonatal pigs ($n = 2$) and 75-, 90-, and 105-d fetal S-V cell culture homogenates (1 culture, 3 treatments/age) by the Trizol method (Life Technologies Inc., Rockville, MD) and applied to RNeasy columns (Qiagen Inc.). For reverse transcription (RT) PCR, 1 μg of total RNA was used for the cDNA synthesis, and 1/40th of that reaction was used as a PCR template (Smith et al., 2003). The following primer sets were used: pig agouti (forward, reverse primer) CCTGTTGGATTTCCTTCTGTC, GGAGGCTTGCAGCTGTTCG and pig 18S (forward, reverse primer) CGGACACGGACAGGATTGAC, TCCACCAACTAAGAACGGCC.

Protein Studies of Adipose Tissue, Serum, and Media Conditioned by S-V Cell Cultures

Adipose Tissue and Media Conditioned by S-V Cell Cultures. Subcutaneous adipose tissue was removed from fetuses and neonatal pigs (5- to 7-d-old) aseptically, and cultures of adipose tissue S-V cells were prepared conventionally (Hausman, 2003). One pool of fetal S-V cells was obtained from 5 to 8 fetuses from each dam, depending on fetal age. We collected and studied 4 pools of S-V cells from 50-d fetuses, 9 pools of S-V cells from 75-d fetuses, 2 pools of S-V cells from 85-d fetuses, 4 pools of S-V cells from 90-d fetuses, and 7 pools of S-V cells from 105-d fetuses. Subcutaneous adipose tissue was removed from additional fetuses at each age, and serum was collected from additional fetuses at 50, 75, 90, and 105 d in utero via the umbilical artery. We studied cultures of S-V cells from each of 7 neonatal pigs (5 to 7 d old; 1 S-V culture = 1 pig) and collected additional subcutaneous adipose tissue from each pig.

Cells (S-V) were conventionally seeded and cultured with or without 80 nM dexamethasone (**Dex**, Sigma Aldrich) until d 3 because early Dex (d 0 to 3) enriches the cultures for preadipocytes by 10- to 20-fold in fetal and young pig S-V cell cultures (Yu et al., 1997). Cultures were switched on d 3 to serum-free medium (Hausman, 2003). Early and late Dex treatment protocols were utilized (Hausman, 2003). Cultures were rinsed once to minimize d 0 to 3 media content before switching to serum-free medium because additional rinses failed to reduce the level of IGF-I or plasminogen activator inhibitor-1 (**PAI-1**) below the level measured after 1 rinse.

Conditioned medium was collected on d 6 of culture (72 h after switching to serum-free medium) in all experiments; in 3 experiments (2 with 75-d and 1 with 105-d fetal cells) media were also collected 144 h after switching to serum-free medium. In several separate experiments with fetal S-V cultures, conditioned medium was collected 2, 6, 12, 24, and 72 h after switching to serum-free medium on d 3. Media were changed every 3 d. Conditioned medium was collected in 50-mL aliquots immediately after plates were taken out of the incubator, and 200 mM phenylmethylsulfonyl fluoride

(**PMSF**) stock (Sigma Aldrich) was added for a final concentration of 1 mM PMSF. Media were frozen at -80°C after the addition of 1 mM PMSF. Fifteen milliliters of S-V cell culture-conditioned media were thawed and concentrated to a final volume of 1 mL by centrifugation at 4°C in 5-kDa molecular weight cut-off centrifuge filters (Amicon, Millipore Corp., Bedford, MA). In attempts to block PAI-1 secretion, 75-d fetal S-V culture media was supplemented with the intracellular protein transport inhibitor, brefeldin (Sigma Aldrich).

Two-Dimensional Fluorescent Differential Gel Electrophoresis. Two dimensional fluorescent differential gel electrophoresis (**DIGE**) was conducted at the Integrated Biotechnology Laboratories following protocols at the University of Georgia (<http://www.prf.uga.edu/index.html>). Samples of media conditioned by young pig S-V control, early Dex, or late Dex cell cultures were prepared, and subsamples of protein extracts were labeled with Cy 2, Cy 3, or Cy 5 fluorescent labels (Amersham Biosciences, Piscataway, NJ). Fifty micrograms of protein from each sample was loaded and isoelectrically focused on 18-cm IPG strips with a pH range of 3 to 10 (Amersham Biosciences) with increasing V from 30 to 4,000 V for 6.5 h followed at 5 W per gel for 1 h and 2 W per gel overnight on $20 \times 26\text{-cm}$, 8 to 15% gradient, polyacrylamide gels. Four 2-dimensional (**2D**) gels with media from 4 separate pig S-V cell culture experiments were run at the same time.

Gel images were obtained using a Typhoon scanner (Amersham Biosciences), and labeled proteins were visualized and quantified with DeCyder 4.0 software (Amersham Biosciences). All detected spots were automatically and manually filtered to exclude spots that did not appear to be protein. Cy 3- and Cy 5-labeled proteins were matched to Cy 2-labeled proteins within a gel, allowing for comparison of protein abundance across samples and gels. Proteins with a 2.5-fold or greater difference in abundance ($P < 0.05$) were considered differentially expressed.

Polyacrylamide Gel Electrophoresis. Protein content of conditioned media and adipose tissue homogenates was quantified using the BioRad protein assay (BioRad Laboratories, Hercules, CA). Equivalent amounts of protein, 50, 100, or 150 μg were loaded on each lane of each gel. Therefore, all comparisons within a 1-dimensional (**1D**) gel were made on an equivalent-loaded protein basis. Media conditioned by fetal and young pig S-V cell cultures and homogenates of adipose tissue from 50-, 75-, and 105-d fetuses and young pigs were electrophoresed on 12.5%, discontinuous, SDS-polyacrylamide gels or Criterion precast gels ($13.3 \times 8.7\text{ cm}$; BioRad Laboratories Inc.) followed by Coomassie blue or silver staining (BioRad Silver Stain Kit, BioRad Laboratories).

Conditioned media from 50-, 75-, and 105-d S-V fetal cultures were compared in each of 4 gels and conditioned media from young pig and later fetal S-V cultures were compared in each of 10 gels. Overall, conditioned media from each of 3 to 5 S-V cell cultures/age were

examined. Adipose tissue homogenates from all fetal age groups were electrophoresed together with 3 to 4 fetuses per age group, for a total of 9 to 11 samples/gel. This was repeated several times with adipose tissue homogenates from additional fetuses (2 to 3) per age group per gel. Adipose tissue homogenates were obtained for 1D PAGE from a total of 10 young pigs.

Visible bands were excised from Coomassie-stained gels, trypsin-digested, and subjected to matrix-assisted, laser desorption ionization-time of flight mass spectrometry (**MALDI-ToF**). The possibility of multiple proteins in a single excised band excluding protein matches was evaluated by removing matching peptide masses from the submitted mass list and searching with the remaining masses. For instance, apolipoprotein (**APO**) E was identified in a sequential extract of young pig adipose tissue upon removal of 1 mass (1442.6864), which is present in APO AIV precursor. The remaining masses were identical to that found in APO E identified in media conditioned by pig S-V cell cultures. Furthermore, the hemoglobin beta chain was identified in conditioned media after removing APO masses. Protein hits with significant protein scores were considered relevant in this study. In these studies, confidence intervals per se were not generated in the analysis.

To consider the possibility of blood-borne protein contamination of adipose tissue homogenates, we examined for hemoglobin in fetal adipose tissue histochemically and biochemically and found no evidence of hemoglobin, indicating no residual blood (Latimer et al., 1993; Hausman et al., 2000). Blood-borne IGF-II was not considered a possible contaminant in studies of IGF-II protein in fetal pig pancreas and muscle (Gerrard et al., 1998; Peng et al., 1996, 1998).

MALDI-ToF and Tandem Mass Spectrometry (MS/MS). Gel spots of interest were excised, de-stained, and subjected to in-gel digestion with trypsin, following protocols developed at the Integrated Biotechnology Laboratories at the University of Georgia (<http://www.prf.uga.edu/index.html>). After overnight digestion with trypsin, the tryptic peptides were extracted with 50% acetonitrile/0.1% trifluoroacetic acid, dried in a Speedvac (Jouan Inc., Winchester, VA), and dissolved in 3 μ L of 50% acetonitrile/0.1% trifluoroacetic acid.

Approximately 25% of each sample was spotted to the MALDI-ToF target. The spotted peptides were mixed (50:50, vol/vol) with a 50%-saturated solution of α -cyano-4-hydroxycinnamic acid (Sigma Aldrich). Peptide mass fingerprinting analysis was carried out using an Applied Biosystems MALDI ToF-ToF mass spectrometer (Foster City, CA) according to standard acquisition parameters at the Integrated Biotechnology Laboratories at the University of Georgia. Samples were analyzed in the positive mode with delayed extraction. The acceleration voltage was set at 20 kV, 1,000 laser shots were summed, and the autolysis products of trypsin, 1045.56 and 2211.096, were used for internal calibration.

The raw MS data for each peptide containing multiple isotope peaks were processed to identify the $^1\text{H}/^{12}\text{C}$ monoisotopic peak for each peptide. Known peptide masses for trypsin autocleavage and human keratin were removed from list of peptide masses, and the resulting peptide masses for a protein were submitted for analysis using the MASCOT search engine (<http://www.matrixscience.com>). The parameters used for searching allowed for searching against all mammalian proteins within the NCBI database and 1 missed-trypsin cleavage site. The mass tolerance was set to 50 ppm. For those spots where peptide mass fingerprinting did not result in a positive identification, MS/MS were acquired on the most abundant tryptic fragments from each spot using standard acquisition parameters. Samples were analyzed in the positive mode using high energy CID (1 kV), 5,000 laser shots that were summed, and default instrument calibration. Twelve spots were submitted for MS/MS. Protein matches with confidence intervals greater than 68% were considered relevant in this study.

Completely Serum-Free S-V Cell Cultures. Adipose tissue S-V cells from young pigs were seeded and maintained in serum-free media (ITS) on either laminin- or fibronectin-precoated dishes (BD Biosciences, Bedford, MA) for 3 d, as previously described (Tchoukalova et al., 2000). Cultures were enriched on d 2 (24 h after seeding), followed by rinsing 3 times and fresh serum-free media (Tchoukalova et al., 2000). Preadipocytes have a stronger affinity for laminin than do fibroblasts, so fibronectin was used as a control substratum. Conditioned media were collected on the third day after seeding. Conditioned media was collected from 4 serum-free, laminin-enriched (preadipocyte) S-V cell cultures (4 pigs) and 2 serum-free, fibronectin-enriched (preadipocytes + fibroblasts) S-V cell cultures.

Cytokine Monoclonal Antibody Arrays. To minimize protein degradation, media were collected, and PMSF was added. Fifteen milliliters of media conditioned by pig S-V cultures were concentrated to a final volume of 1 mL by centrifugation ($2,000 \times g$) at 4°C in 5-kDa molecular weight cut-off centrifuge filters (Amicon, Millipore Corp.). Media from 4 S-V cultures treated with early Dex as well as conditioned media from 2 control (no Dex) treated S-V cultures were examined. Each conditioned media cytokine array represented media from a different cell culture. In addition, media from the 4 cultures used in the 2D DIGE (MALDI-ToF) study was combined and examined in 2 cytokine arrays. Each serum cytokine array represented serum from a different pig, and 1 mL of serum was used.

Cytokine monoclonal antibody arrays, Ray Bio Human Cytokine Antibody Array VI (cat # H0108006) and Array VII (cat # H0108007) were used as directed (Ray-Biotech Inc., Norcross, GA). Briefly, nonspecific binding was blocked by incubation with a blocking buffer for 30 min at room temperature. Arrays were incubated with samples for 1 h at room temperature. Array membranes were probed with a mixture of biotin-labeled antibodies

recognizing each cytokine for 1 h at room temperature. This was followed by a 1-h incubation with labeled streptavidin, which was visualized using a chemiluminescent reagent system. Protein density was determined with a densitometer (Storm 860, Molecular Dynamics, Sunnyvale, CA) and computer-assisted image analysis (Image-Pro Plus, Media Cybernetics Inc., Silver Spring, MD).

Plasminogen Activator Inhibitor-I Assays. The PAI-1 concentrations in media conditioned by S-V cell cultures were determined using an enzyme-linked immunosorbent assay that measured free and complexed porcine PAI-1, similar to the protocol described by Crandall et al. (1999). A human PAI-1 monoclonal antibody that strongly cross reacts with porcine PAI-1 was used (Debrock and Declerck, 1997). Interassay CV was 8%.

IGF Binding Protein Ligand Blot Assays and IGF-I and -II RIA. As reported (Hausman et al., 2002), conditioned media proteins from 85- and 105-d fetal S-V cultures were electrophoresed on 12.5% discontinuous SDS-polyacrylamide gels. Proteins were transferred to Immobilon-P membranes (Amicon, Millipore Corp.), and blots were incubated with ^{125}I -IGF-I and then exposed to film for 8 to 10 d. Protein bands [IGF binding protein (IGFBP)] were visualized by autoradiography, and molecular weight was estimated by comparison to prestained protein standards.

As reported (Hausman et al., 2002), IGF-I RIA was performed using rabbit IGF-I antiserum. The intra- and interassay CV were 5.4 and 6.6%, respectively. Samples were assayed for IGF-II using recombinant human IGF-II as standard and tracer, and rabbit anti-human IGF-II polyclonal antiserum (Hausman et al., 2002). Intra- and interassay CV were 5.5 and 7.2%, respectively.

Statistics

Concentrations of PAI-1, IGF-I, and IGF-II in fetal S-V culture conditioned media were analyzed by a 2-way ANOVA for main effects of fetal age and Dex treatment using PROC GLM (SAS Inst. Inc., Cary, NC). The main effect of dam per se was not determined because cell culture media treatments were not duplicated within a litter. As a result, each fetal S-V cell culture represented a separate dam for all measurements. Concentrations of PAI-1 in young pig S-V culture conditioned media were analyzed by 1-way ANOVA for the main effect Dex treatment using PROC GLM (SAS Inst. Inc.). Differences between means were determined by the least squares contrasts using the PROC GLM procedure.

RESULTS

Gene Expression Studies of Neonatal Adipose Tissue and Fetal S-V Cell Cultures

Microarray Studies. Our microarrays were designed for studies of several tissues including ovary, uterus,

pituitary, hypothalamus, and adipose. A total of 200 and 160 genes were expressed 40-fold over basal intensity levels in fetal S-V culture and adipose tissue microarrays, respectively. Eleven percent of genes in both S-V culture and adipose tissue microarrays had normalized intensities ≤ 400 , whereas normalized intensities of 25 apparently unrelated genes averaged 150 ± 20 for S-V culture arrays and 200 ± 40 for adipose tissue arrays. Because our microarrays were designed for study of other tissues in addition to adipose tissue, they contain genes that can be considered unrelated controls because they are primarily expressed by another tissue or group of tissues. For instance, normalized intensity units ranged from 71 to 390 in fetal and young pig adipose tissue microarrays for the pituitary genes preprolactin and thyroid stimulating hormone beta, the hypothalamic genes somatostatin and melanin concentrating hormone, and preproinsulin, a pancreas gene. Furthermore, a study of 5 tissues in growing pigs with our microarrays showed that preprolactin and thyroid stimulating hormone beta were significantly expressed only in pituitary microarrays, and somatostatin and melanin concentrating hormone were significantly expressed only in hypothalamus microarrays (G. J. Hausman, unpublished observations). Intensity units in fetal and young pig adipose tissue microarrays ranged from 50 to 200 for other examples of unrelated control genes including parathyroid hormone receptor 1, prepronociceptin, and secreted neuronal and endocrine protein (7B2) mRNA, which was expressed in a tissue specific manner in microarray studies of growing pigs (G. J. Hausman, unpublished observations). It should be noted that the average background intensity was 340 ± 42 for neonatal adipose tissue microarrays and 265 ± 6 for fetal S-V cell culture arrays, and the overall average of lambda DNA controls in adipose tissue and S-V culture microarrays was 1.3 intensity units. Despite a limited number of replicates, expression of the IL-1 beta precursor and putative preproadipsin were significantly decreased in adipose tissue arrays from young pigs relative to fetal adipose tissue arrays (significant *t*-test of the normalized intensity means; $P > 0.05$). In general, gene expression patterns for adipose tissue and fetal S-V culture arrays were remarkably similar. Relative expression of IGF-I and II, IL, interferons, and IGFBP-3 in adipose tissue and fetal S-V culture microarrays are shown in Table 1. Expression of known adipocyte or adipose tissue secreted factors included PAI-1; tumor necrosis factor (TNF)- α ; transforming growth factor- β 1; nerve growth factor; IGF-I, -II; IGFBP-2,-3,-5,-6; IL-4, -6,-15, -1 β ,- 1A; and interferon alpha 2 (Table 2). Genes expressed with no previous report of adipose tissue expression included relaxin, insulin-like 3, chromogranin A, brain-derived neurotrophic factor (BDNF), and IL-12 (Table 3).

Agouti RT-PCR Studies. Neonatal adipose tissue clearly expressed agouti mRNA, as did fetal S-V cell cultures (Figure 1). Agouti mRNA was expressed by

Table 1. Relative expression of IGF-II, IL-4, IL-1 α , IGF binding protein (IGFBP)-5, interferon gamma (IFNG), and interferon alpha 6 (IFNA6) in neonatal adipose tissue and fetal stromal-vascular (S-V) cell culture microarrays¹

Gene	Neonatal adipose tissue	Fetal S-V cell culture	Gene	Neonatal adipose tissue	Fetal S-V cell culture
IGF-I	100	100	IGFBP-2	100	100
IGF-II	290 \pm 70	305 \pm 60	IGFBP-5	135 \pm 20*	300 \pm 6
IL-15	100	100	IFNB1	100	100
IL-4	54 \pm 3*	86 \pm 6	IFNG	32 \pm 1*	59 \pm 16
IL-1 α	34 \pm 4	49 \pm 23	IFNA6	16 \pm 1	35 \pm 14

¹Expression level percentages [levels of IGF-I, IL-15, IGFBP-2, and interferon beta (IFNB) 1 were arbitrarily set at 100] included IGF-II/IGF-I, IL-4/IL-15, IL-1 α /IL-15, IGFBP-5/IGFBP-2, IFNG/IFNB1, and IFNA6/IFNB1. Means \pm SEM of 4 percentages of relative normalized fluorescent intensities from each of the 4 neonatal adipose tissue arrays (representing 2 fetuses + 2 young pigs), and means \pm SEM of 2 percentages from each of the 2 fetal S-V culture arrays.

* $P < 0.05$, neonatal adipose tissue vs. fetal S-V cell cultures, Student's *t*-test.

fetal S-V cell cultures with and without Dex treatment (Figure 1).

Protein Studies of Adipose Tissue, Serum, and Media Conditioned by S-V Cell Cultures

One-Dimensional PAGE and MALDI-ToF Studies. Several significant ($P < 0.05$) identifications for APO-A1 protein were obtained for a 30-kDa protein from several Coomassie-stained gels of adipose tissue from 105-d fetuses indicating the presence of APO-A1 (Table 4 and Figure 2). The APO-A1 was also present in serum and younger fetal tissue based on significant ($P < 0.05$) identifications for APO-A1 obtained for a 30-kDa band in gels of 75-d fetal serum and 50-d fetal adipose tissue (Table 4 and Figure 2). The APO-A1 band in fetal adipose tissue comigrates with the APO-A1 band in fetal serum (Figure 2). An APO-A1 band was also evident in gels of adipose tissue from young and older pigs (not shown). We failed to obtain significant protein identification for the 33-kDa band despite high quality spectra. A 30-kDa band was not detected in Coomassie gels of pig placenta (not shown).

A predominant string of protein spots in 2D gels of conditioned media (Figure 3) was remarkably similar to strings of APO-A1 spots in published 2D gels of plasma, lymph, HEPG2 secreted proteins, fibroblast conditioned media, and adipose tissue (Sanchez et al., 1995; Sanchez et al., 2001; Lim and Bodnar, 2002; Corton et al., 2004; Leak et al., 2004). A similar string of proteins was observed in 2D gels of media conditioned by S-V cell cultures that were cultured on either laminin or fibronectin and never exposed to serum (Figure 3B, representative of 4 pig S-V culture gels). A major 30-kDa protein band in 1D Coomassie-stained polyacrylamide (12.5%) gels of conditioned media corresponded to this string of protein spots in 2D gels (Figure 3). This band was excised from Coomassie-stained gels of media conditioned by fetal and pig S-V cultures, digested using trypsin, and identified as APO-A1 using MALDI-ToF mass spectrometry and database searches (Table 4).

One- and 2-dimensional PAGE showed that the concentration of APO-A1 (30 kDa band intensity and size) in media conditioned by fetal S-V cells increased with fetal age and with time in culture (not shown). A larger APO-A1 band (30 kDa) was detected in conditioned media after Dex treatment in 105-d fetal S-V cultures (Figure 2; 2 fetal S-V cultures, representing 2 dams, were examined and results replicated on 3 gels). The influence of differentiation on APO-A1 levels was not examined in S-V cell cultures from any other age. Regardless of treatment, a 30-kDa band was discernible in all gels of media conditioned by pig and 75-d and older fetal S-V cultures.

We examined greater and lower molecular weight bands from the conditioned media gels used to identify the 30-kDa APO-A1 band and obtained significant ($P < 0.05$) identifications for APO-AIV protein for a 43-kDa band and significant ($P < 0.05$) identifications for APO-E for a 36-kDa band (Table 4). The location of the excised APO-E (36 kDa) band is depicted in Figure 2 because the same conditioned media was run in this gel (Figure 2) as was run in the gel used to excise the band. An APO-E (36 kDa) band was only detected in conditioned media after Dex-induced adipocyte differentiation in 75- and 105-d fetal S-V cultures (Figure 2). Surprisingly, examination of a lower protein band in a conditioned media gel yielded significant identifications ($P < 0.05$) for porcine hemoglobin beta chain [P02067, molecular weight (MW) 16,034, protein score, 83]. Apolipoprotein-E was also identified in a band excised from a gel of adipose tissue proteins from a young pig (Table 4).

2D DIGE, MALDI-ToF, and MS/MS Studies. Although similar protein concentrations were loaded, there were fewer total protein spots in 2D gels from control media 2D gels than from other treatments precluding comparison of control media protein spots to Dex-treated media spots. DeCyder image analysis of Dex treatment images detected an average of 864 ± 31 (mean \pm SEM of 4 gels) spots per 2D gel. And, comparison of late Dex to early Dex treatment showed that the

Table 2. Secreted factors expressed in neonatal adipose tissue and fetal stromal-vascular cell culture microarrays with previous reports of adipose tissue expression

Classification	Acc# TIGR ¹	Identification
Growth-related	TC183976	Insulin-like growth factor II precursor (IGF-II) ²
	TC185594	Insulin-like growth factor I precursor (IGF-I) ²
	TC191294	Fibroblast growth factor 7 (FGF-7)
	TC174734	Acidic fibroblast growth factor (a FGF)
	NP276134	
	Singleton	Insulin-like growth factor binding protein 5 (IGFBP-5) ²
	NP275375	
	Singleton	Insulin-like growth factor binding protein 3 (IGFBP-3) ²
	NP275499	
	Singleton	Insulin-like growth factor binding protein 2 (IGFBP-2) ²
	TC184281	Insulin-like growth factor binding protein 6 (IGFBP-6)
Cytokine	NP277067	
	Singleton	Nerve growth factor B (NGFB)
	TC184148	Tumor necrosis factor precursor (TNF-alpha) ²
	NP276238	
	Singleton	Transforming growth factor beta-1 (TGF-beta1) ²
	TC10365	Interleukin-1 alpha (IL1 α) ²
	TC162694	Interleukin-1 beta precursor (IL-1 beta)
	TC170828	Interleukin-4 (IL-4) ²
	TC170420	Interleukin-5 (IL-5)
	TC34424	Interleukin-6 (IL-6) ²
	TC186938	Interleukin-15 precursor (IL-15)
	TC12976	Interferon, alpha 6 (IFNA6)
	TC162838	Interferon gamma precursor (IFN-gamma)
	TC192476	Interferon-beta-1 precursor (IFN-beta1)
	NP276007	
	Singleton	Transforming growth factor alpha (TGF-alpha)
	TC20146	Angiopoietin 2 (ANGPT2) ²
	TC13475	Tissue inhibitor of metalloproteinase 1 (TIMP1) ²
	TC163286	Small inducible cytokine A2 precursor (CCL2)
Hormone	TC187888	Follitropin beta chain precursor (FSH beta subunit)
	TC161970	Lutropin beta chain precursor (LHbeta subunit)
	NP276761	
	Singleton	Corticotropin releasing hormone (CRH)
	TC162733	Follistatin precursor (Follistatin) ²
Other	TC181439	Apolipoprotein-E (Apo-E) ²
	TC200187	Apolipoprotein A-I precursor (Apo-A1) ²
	TC164742	Apolipoprotein A-IV (Apo-IV) ²
	NP275923	
	Singleton	Plasminogen activator inhibitor I (PAI-1) ²
	TC 233927	Agouti signaling protein ²
	TC172583	Agouti-related protein (AGRP)
	TC186115	Leptin
	TC21820	Putative preproadipsin
	TC12140	Matrix metalloproteinase 1 (MMP1)
	TC199414	Alpha-1 acid glycoprotein (fragment)

¹TIGR (The Institute of Genome Research), TC, and NP accession numbers can be searched at: http://www.tigr.org/tigr-scripts/tgi/T_reports.cgi?species=pig

²Also identified in either proteomic or conventional protein assays.

quantity of $98 \pm 1\%$ of the spots was not changed (means \pm SEM of 4 gels). Eighty-six spots common to both Dex treatments that were 2.5 fold or greater ($P < 0.05$) in quantity compared with controls (image analysis) were selected for further study. Forty-four of the 86 spots had high quality spectra, but only 34 had protein scores that allowed tentative identification. Of these 34, 12 spots were of sufficient quantity for MS/MS analysis.

Several of the larger proteins typically present in media conditioned by S-V cultures were identified and verified by MS/MS analysis (Table 5). In this study the low level of protein in most spots precluded MS/MS verification of most proteins. Nevertheless, several secreted proteins identified with MALDI-ToF analysis with confidence intervals (CI) > 68 were also identified in either gene microarray or protein array studies. These included:

Table 3. Secreted factors expressed in neonatal adipose tissue and fetal stromal-vascular cell culture microarrays with no previous reports of adipose tissue expression

Classification	Acc# TIGR ¹	Identification
Growth-related	TC164670	Relaxin (Relaxin) ²
	TC165299	Insulin-like 3 precursor (INSL3)
	TC167241	Fibroblast growth factor 12, isoform B (FGF-12)
	TC15832	Insulin-like growth factor binding protein 7 (IGFBP-7)
	NP282998	
Cytokine	Singleton	Growth differentiation factor (GDF9B)
	TC186429	Brain-derived neurotrophic factor (BDNF) ²
	NP276182	
	Singleton	Interleukin-12 (IL-12) ²
	TC169480	Short type I interferon precursor (spI IFN)
Other	TC16303	Apolipoprotein R precursor (Apo-R)
	TC163235	Chromogranin B (CHGB)
	TC187206	Chromogranin A precursor (CHGA)
	TC183472	Glycoprotein hormones alpha chain precursor

¹TIGR = The Institute of Genome Research.²Also identified in either proteomic or conventional protein assays.

IGFBP-5 (Q28985, MW 30,337, CI 90, 95%) IL-8 precursor (P36925, MW 11,292, CI 69%), follistatin (P50291, MW 37,959, CI 73%), BDNF (P14082, MW 28,287, CI 82, 83%), and relaxin (P11185, MW 6,071, CI 56, 92%). Several proteins not identified by gene microarrays or protein arrays included heparin-binding epidermal growth factor (**EGF**) like-factor (**HBEGF**; Q01580, MW 22,985, CI 60, 85%), cyclophilin B (P80311, MW 22,701,

CI 92%), and phosphomevalonate kinase (**PMKASE**; Q29081, MW 14,705, CI 98, 100%). Identification of porcine proteins in this group of proteins included BDNF, HBEGF, IGFBP-5, and PMKASE. Porcine proteins identified with weaker or lower scores included endothelin-1 precursor (P09558, MW 23,243, CI 63%).

Unidentified Secreted Proteins. The 2D DIGE study of media conditioned by pig S-V cell cultures identified 10 proteins with high quality mass spectra but no matches in any database. Two proteins with high quality mass spectra but no database matches were identified in the 1D gel study of media conditioned by fetal and pig S-V cell cultures. Therefore, a total of 12 adipocyte secreted proteins remain to be identified.

Cytokine Monoclonal Antibody Array Studies. Segments of VI and VII cytokine arrays of serum and media conditioned by pig S-V cell cultures are shown in Figure 4. Because these are human monoclonal cytokine arrays, pig serum immunoreactivity was used to indicate or validate porcine reactivity for a given cytokine. Many of the monoclonal antibodies did not crossreact with porcine serum cytokines, whereas some did (Figure 4). Reactivity for 24 cytokines was consistently detected (across the 3 to 4 arrays used) in both conditioned media and serum arrays (Figure 4). Array segments are not shown for 2 of the 24 cytokines detected in serum and conditioned media i.e., fibroblast growth factor (**FGF**)-6 and Fas (CD95/APO1)/TNFR. Twelve of the 24 cytokines were also identified in either the gene microarray or the 2D DIGE study i.e., IL-12 p40, IL-6, IL-4, IL-3, IL-1 α , IL-8, angiopoietin (**ANGPT**)-2, TNF α , BDNF, bone morphogenetic protein (**BMP**)-4, tissue inhibitor of metalloproteinase (**TIMP**)-1,-2, and IGFBP-3 (Figure 4). The other cytokines detected in conditioned media and serum include IL-1ra, IL-16, IL-6R, small inducible cytokine A5

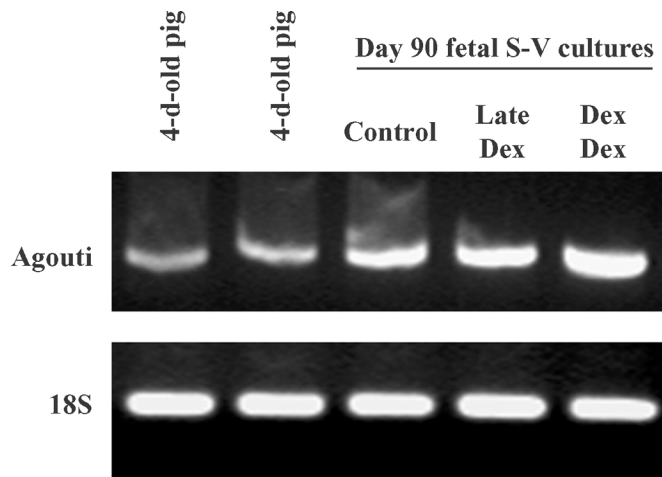


Figure 1. Reverse transcription-PCR (RT-PCR) analysis of agouti mRNA in adipose tissue and fetal stromal-vascular (S-V) cell cultures. Total RNA was isolated from subcutaneous adipose tissue from 4-d-old pigs (n = 2) and 90-d fetal S-V cell culture homogenates (1 culture, 3 treatments/age). Culture treatments included controls, late dexamethasone (Dex), and Dex treatment throughout culture (Dex, Dex). For RT-PCR, 1 μ g of total RNA was used for the cDNA synthesis, and 1/40th of that reaction was used as a template for 35 cycles of PCR.

Table 4. Proteomic analysis of stromal-vascular (S-V) cell culture-conditioned media and adipose tissue:protein hits with significant scores

Protein ¹	Source	Accession No.	Molecular mass (Da)	pI	Protein score	Species
APO-A1	75-d fetal serum	P18648	30,325	5.48	195	<i>Sus scrofa</i>
APO-A1	50- and 105-d fetal adipose tissue	P18648	30,325	5.48	192, 182, 156	<i>Sus scrofa</i>
APO-A1	Fetal and pig S-V-conditioned media	P15497	30,276	5.71	184, 204	<i>Bos taurus</i>
		P18648	30,325	5.48		<i>Sus scrofa</i>
APO-E	Fetal S-V-conditioned media and pig adipose tissue	P18650	36,599	5.62	92	<i>Sus scrofa</i>
APO-AIV	Pig S-V conditioned media	CAA11020	43,294	5.69	177	<i>Sus scrofa</i>

¹APO = apolipoprotein.

(**RANTES**), vascular endothelial growth factor (VEGF), VEGF-D, TNF- β , TNF-related apoptosis inducing ligand receptor 4 (**TRAIL-R4**), and urokinase plasminogen activator surface receptor (**uPAR**; Figure 4). Therefore, cytokine arrays enabled us to identify 24 cytokines in media conditioned by pig S-V cell cultures. Reactivity for platelet derived growth factor (**PDGF**)-BB and adiponectin was greater in serum arrays than in conditioned media arrays (Figure 4). Therefore, PDGF-BB and to lesser extent, adiponectin, was undetectable in conditioned media (Figure 4). Patterns of cytokine immunoreactivity in VI arrays of individual (Figure 4) and combined media (2D DIGE study) were indistinguishable. Combined media from 2D DIGE was also analyzed to link the cytokine array and 2D DIGE results.

The raw image analysis data (volume) was corrected by subtracting the blank from the positive controls, and the negative controls were subtracted from the volume values for each spot. Ratios of the spot volume/positive

control were calculated, and the least squares means \pm SEM of ratios for cytokines in conditioned media arrays with the greatest ratios included TIMP-2, 1.63 ± 0.45 ; VEGF, 1.42 ± 0.5 ; IL-8, 1.12 ± 0.45 ; TRAIL-R4, 1.1 ± 0.45 ; and uPAR, 0.9 ± 0.5 .

PAI-1 ELISA Studies. Treatment from d 1 to 3 (72 h) with brefeldin at $1 \mu\text{g/mL}$ reduced PAI-1 levels to 9 to 61% of the PAI-1 levels in control (no drug) 75-d fetal S-V cultures ($n = 2$ studies). Treatment from d 1 to 3 with brefeldin at $1 \mu\text{g/mL}$ followed by no drug for 2 to 4 d reduced PAI-1 levels to 72 to 76% of control culture levels ($n = 2$ studies), whereas continuous treatment with $0.5 \mu\text{g/mL}$ did not reduce PAI-1 levels (i.e., 116 to 88% of controls). Concentrations of PAI-1 in 75-d fetal S-V cultures peaked at 72 h and increased by 230 to 175% between 24 and 72 h of culture ($n = 2$). Rinsing 2 to 3 times before switching to serum-free media (ITS) was examined in 1 study and found to have little effect on PAI-1 secretion, and PAI-1 was not detectable in the medium alone. Rinsing several times before switching

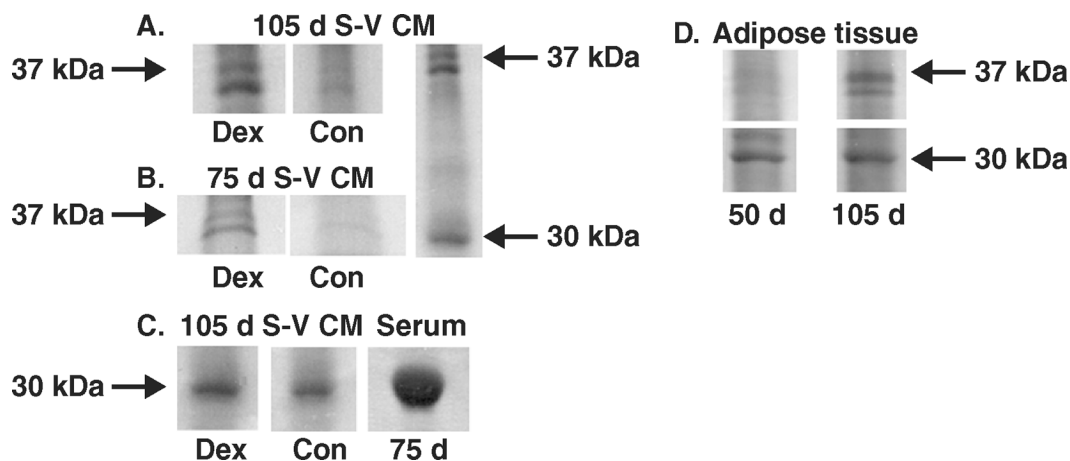


Figure 2. Coomassie-stained 30-kDa apolipoprotein (APO)-A1 and 37-kDa (APO-E) bands in SDS-polyacrylamide gels of conditioned media from 75-d [B, 75-d stromal-vascular (S-V) conditioned media (CM)] and 105-d (A and C, 105-d S-V CM) fetal S-V cultures, serum from a 75-d fetus (C), and adipose tissue from 50- and 105-d-old fetuses (D). Media conditioned from d 3 to 6 of culture were collected and concentrated. Note that dexamethasone (Dex) treatment increased both the APO-E band (37 kDa) in 75- (B) and 105-d (A) fetal S-V culture media and the APO-A1 band (30 kDa) in 105-d (C) fetal S-V culture media. Note that fetal age did not influence the APO-A1 band (30 kDa) in adipose tissue gels, whereas the 37-kDa protein band increased with fetal age (D). Also note the paucity of significant protein bands above 30 kDa in 105-d fetal S-V culture media.

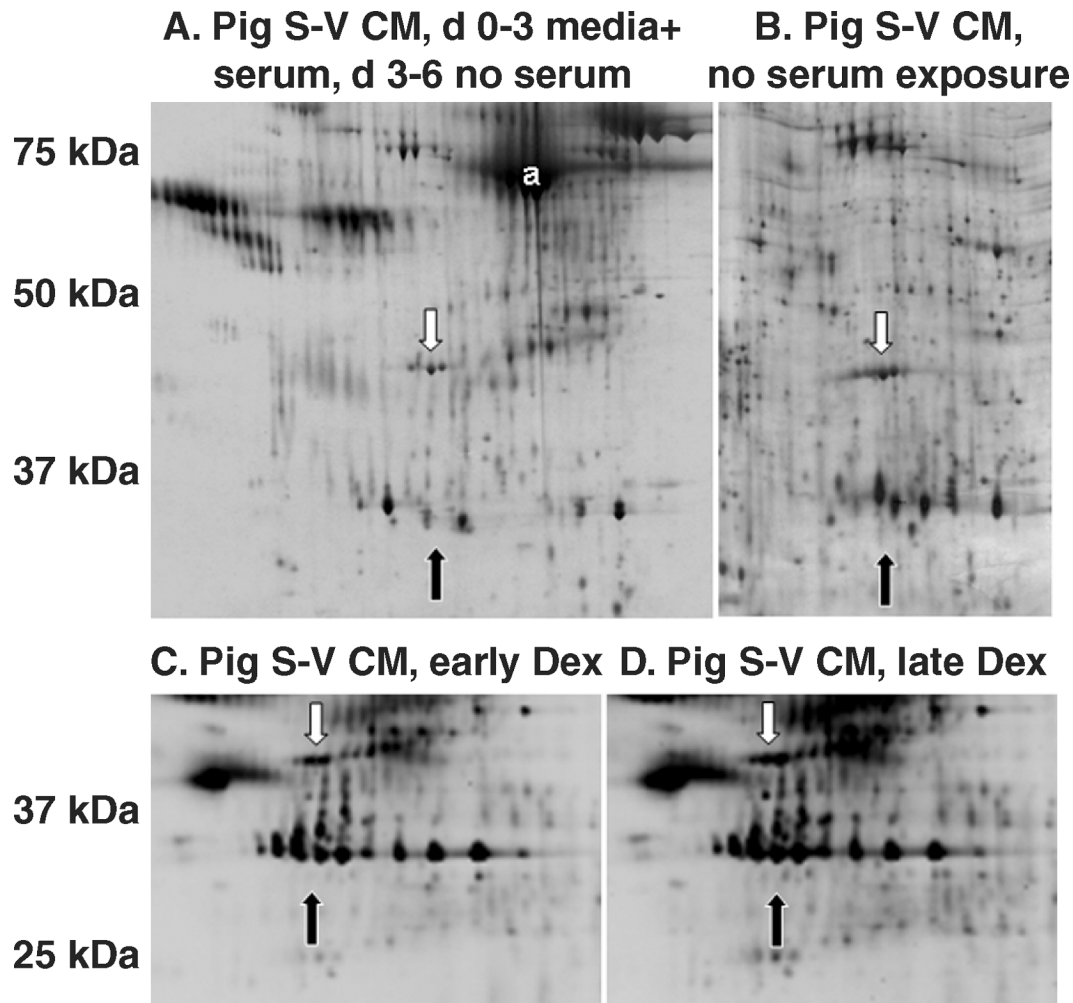


Figure 3. Two-dimensional electrophoretograms of conditioned media (CM) from d 3 to 6 (A, C, D) or from d 1 to 3 (B) and from stromal-vascular (S-V) cell cultures from young pigs. (A) control cultures, no dexamethasone (Dex), (C) late Dex-treated cultures, (D) early Dex-treated cultures, and (B) cultures never exposed to serum. Fifty micrograms of concentrated, conditioned media protein was run at 110 mA/gel in isoelectric focusing tube gels, extruded into equilibration buffer, and electrophoresed in 10% acrylamide slab gels. Gels were fixed and silver stained. Results shown in C and D and results shown in A and B should be considered independently because they represent different experiments. Note that serum-free conditions (B) did not adversely influence the size of the 30-kDa string of protein spots (A and B, black arrows) and a smaller string of protein spots of greater molecular weight (A and B, white arrows) despite the reduction in larger serum-type proteins such as albumin (A, a). Also note a similar distribution and number of protein spots in electrophoretograms of media from early Dex- and late Dex-treated cultures (C vs. D). In particular, the 30-kDa string of protein spots (C and D, black arrows) and a smaller string of protein spots of greater molecular weight (C and D, white arrows) were similar in electrophoretograms of media from early and late Dex-treated cultures (C, D).

to serum-free media also had little effect on IGF-I secretion in fetal pig S-V cultures (Hausman et al., 2002).

There was no influence ($P > 0.34$) of early or late Dex treatment on PAI-1 concentrations (ng of PAI-1/ng of total protein) in media conditioned by either young pig cultures ($n = 8$ cell cultures) or by fetal S-V cultures from 50-, 75-, 90-, and 105-d fetuses [$n = 3$ to 4 cell cultures (cell pools)/ age]. Concentrations of PAI-1 in conditioned media from fetal S-V cultures were not influenced ($P > 0.23$) by fetal age [$n = 3$ to 4 cell cultures (cell pools)]. The PAI-1 concentrations (ng of PAI-1/

ng of total protein) in media conditioned by young pig cultures were 203 ± 42 for control cultures (F – ITS) and 241 ± 42 for late Dex-treated cultures ($n = 4$ cell cultures). When not adjusted for protein, the control culture media PAI-1 concentration was 355 ± 12 ng/mL, which was similar to the PAI-1 level of 742 ± 144 ng/mL detected in serum from market weight pigs ($n = 4$).

Insulin-Like Growth Factor-I and -II RIA and IGFBP Ligand Blot Studies. As expected, all 4 major IGFBP were detected in ligand blots of media condi-

Table 5. Proteomic analysis of young pig stromal-vascular cell culture-conditioned media; protein hits with 100% confidence intervals (CI)¹

Protein	Accession No.	Molecular mass (Da)	pI	Sequence coverage, %	Species	Mascot score	No. matching peptides
Serotransferrin ²	Q29443	77,753	6.75	21	<i>Bos taurus</i>	126	14
Fetuin ²	P12763	38,418	5.26	35	<i>Bos taurus</i>	109	8
Alpha-1-antiproteinase inhibitor	P34955	46,103	6.05	32	<i>Bos taurus</i>	172	13
Serum albumin precursor ^{2,3}	P02769	69,293	5.82	34	<i>Bos taurus</i>	221	18
Actin, (beta actin)	P29751	41,755	5.3	29	<i>Bos taurus</i>	124	8
Serine/threonine protein phosphatase 2A ⁴	P54612	65,191	5	9	<i>Sus scrofa</i>		4
Collagen alpha 1(I) chain precursor	Q9XSJ7	138,677	5.6	10	<i>Canis familiaris</i>	76	9
Collagen alpha 2(I) chain precursor	O46392	129,321	9.8	8	<i>Canis familiaris</i>	84	10

¹Selected spots or proteins were common to conditioned media from both Dex treatments.

²Also tandem mass spectrometry (MS/MS) hits.

³High CI were obtained for *Ovis aries* serum albumin precursor (CI, 99,99; P14639) and for *Sus scrofa* serum albumin precursor fragment (CI 90,98; P08835).

⁴Only identified by MS/MS, so a Mascot score was not generated.

tioned by fetal (85 and 105 d) and young pig S-V cells cultured under basal conditions (data not shown). Concentrations of IGF-I were greater ($P < 0.05$) in media (ITS) from 105-d fetal S-V cultures than in media (ITS) from 85-d fetal S-V cultures (8.4 ± 0.3 and 3.3 ± 0.4 , ng/mL, respectively; means \pm SEM of 3 to 4 cultures). Concentrations of IGF-II were similar in media conditioned by control 85- and 105-d fetal S-V cultures (111 ± 11 and 135 ± 14 ng/mL, respectively; means \pm SEM of 3 cultures).

Regardless of treatment, concentrations of IGF-I in media conditioned by 105-d S-V cultures from d 6 to 9 decreased ($P < 0.05$) by $58 \pm 5\%$ compared with IGF-I concentrations in media conditioned from d 3 to 6.

DISCUSSION

In the current study we report the first examination of proteins secreted by pig adipose tissue utilizing a global approach that included a combination of gene microarrays, proteomics, antibody arrays, and conventional protein assays. The current study is also the first and only gene microarray and/or proteomic study of neonatal adipose tissue. Comparing the gene microarray results to the results of proteomic and protein assays indicated that a group of 20 adipose tissue-secreted proteins were detected or identified by both approaches. This group of secreted proteins is remarkably diverse and includes cytokines, growth factors, and APO. Many of these proteins have been identified previously as adipocyte secreted factors by gene microarrays, proteomics, or conventional gene and protein approaches (reviews, Hausman et al., 2001; Trayhurn and Wood, 2004; Gimeno and Klamann, 2005; Hauner, 2005). These include IGF-I, II, IGFBP-5, -3, -2, IL-1, -4, -6, transforming growth factor- β and TNF α . However, some of these adipose tissue secreted proteins have not been examined or identified in the pig or any meat animal species, including PAI-1, ANGPT-2, TIMP-1, IL-4, IL-1 α and APO-A1, APO-AIV, and APO-E. Fur-

thermore, BDNF, relaxin, and IL-12 have never been reported as adipose tissue-secreted proteins. The detection of IL-12 and BDNF is possibly attributable to the presence of macrophages because neonatal adipose tissue and S-V cell cultures would have relatively greater numbers of macrophages than mature adipose tissue and S-V cell cultures. Based on published gene microarray and proteomic studies, this is the very first report that relaxin may be secreted by adipose tissue. Although novel, this finding seems to have little apparent significance to adipose tissue biology.

Cytokine antibody array and 2D DIGE studies detected IL-8 protein in conditioned media, which is consistent with the evidence that IL-8 is one of the primary cytokines secreted by adipose tissue (reviews, Trayhurn and Wood, 2004; Gimeno and Klamann, 2005; Hauner, 2005). Regardless, this is the first evidence that IL-8 is an adipose tissue-secreted factor in the pig or any meat animal species. We did not examine IL-8 gene expression because oligonucleotide probes for IL-8 were not spotted in our microarrays.

Adipose tissue agouti gene expression has been demonstrated in bovines (Sumida et al., 2005) and humans (Xue and Zemel, 2000; Smith et al., 2003). This study provides the first evidence that agouti and agouti-related protein mRNA is expressed by pig adipose tissue and S-V cell cultures. Agouti is secreted by adipose tissue and was the first obesity gene cloned and characterized (review, Kim and Moustaid-Moussa, 2000). In vitro studies and studies of agouti overexpression in mouse adipose tissue show that agouti increases PPAR γ expression and adipogenesis (Mynatt and Stephens, 2001; 2003). Agouti strongly antagonizes several melanocortin receptors, which have been detected in mouse and human adipose tissue (Smith et al., 2003). Therefore, it is likely that agouti influences porcine adipogenesis in an autocrine/paracrine manner.

We identified several large serum-borne proteins in S-V cell culture-conditioned media including serotransferrin, fetuin, albumin, and alpha-1-antiproteinase in-

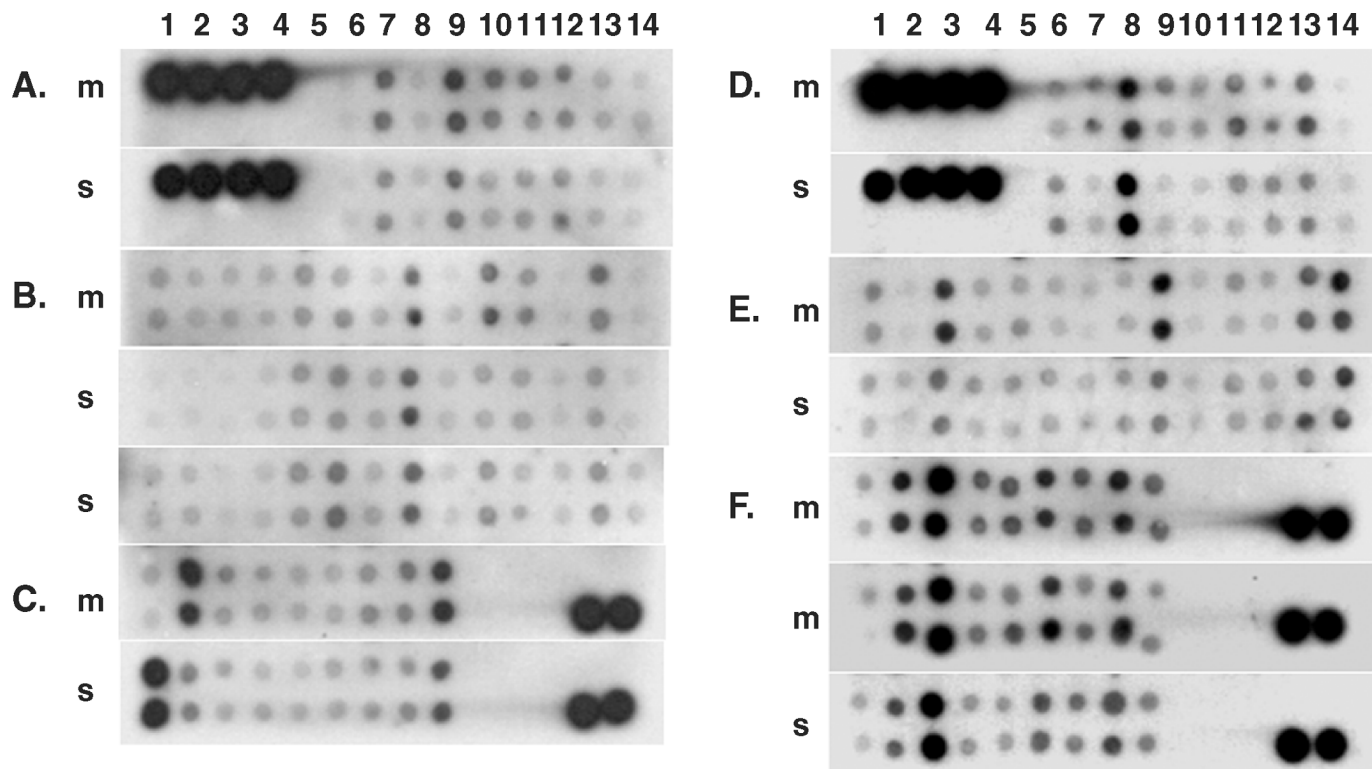


Figure 4. Strips of cytokine monoclonal antibody arrays incubated with stromal-vascular (S-V) cell culture-conditioned media (m) or with pig serum (s). Arrays were incubated with 1 mL of concentrated conditioned media or 1 mL of undiluted pig serum for 1 h at room temperature and then probed with a mixture of biotin-labeled antibodies recognizing each cytokine for 1 h at room temperature. This was followed by a 1-h incubation with labeled streptavidin, which was visualized using chemiluminescent reagents. Note the similar patterns of cytokine immunoreactivity for m and s samples. Also note the similar patterns of reactivity for serum from 2 different pigs (B, s,s) or media from 2 different S-V cultures (F, m,m).¹ Spot identities were as follows:

Location	Spot identity	Location	Spot identity	Location	Spot identity	Location	Spot identity
A - 1-4	+ controls	B - 10	IL-3	D - 1- 4	+ controls	F - 3	TIMP-2
A - 5	blanks	B - 11	IL-4	D - 5	blank	F - 5	TRAIL R3
A - 7	BDNF	B - 13	IL-6	D - 6	adiponectin	F - 6	TRAIL R4
A - 9	BMP-4	C - 1	PDGF-BB	D - 8	ANG-2	F - 7	uPAR
A - 10	BMP-6	C - 2	Rantes	E - 3	IGFBP-3	F - 8	VEGF
A - 12	CNTF	C - 8	TNF- α	E - 9	IL-12 p40	F - 9	VEGF-D
B - 5	IL-16	C - 9	TNF- β	E - 13	IL-6R	F - 10-11	blanks
B - 6	IL-1 α	C - 10-11	blanks	E - 14	IL-8	F - 13-14	+ controls
B - 8	IL-1ra	C - 13-14	+ controls	F - 2	TIMP-1		

¹Abbreviations: CNTF = ciliary neurotrophic factor; ANG = angiopoietin; BDNF = brain-derived neurotrophic factor; BMP = bone morphogenetic protein; IL-1ra = IL-1 receptor agonist; PDGF = platelet-derived growth factor; TNF = tumor necrosis factor; TIMP = tissue inhibitor of metalloproteinase; TRAIL = TNF-related apoptosis inducer ligand; uPAR = urokinase plasminogen activator surface receptor; and VEGF = vascular endothelial growth factor.

hibitor. These serum borne proteins were also identified in several proteomic studies of serum-free media conditioned by fibroblasts (Lim and Bodnar, 2002; Prowse et al., 2005). The presence of these proteins was largely attributed to previous exposure to fetal bovine serum despite extensive rinsing after removal of serum containing media (Lim and Bodnar, 2002; Prowse et al., 2005). However, it is important to consider that sero-

transferrin was identified as an adipocyte-secreted factor in a proteomic study of media conditioned by rat adipocytes never exposed to serum (Chen et al., 2005). Another large serum borne protein, alpha-fetoprotein, and albumin were also detected in adipocyte-conditioned media (Chen et al., 2005). Their approach involved 2D liquid chromatography separation in lieu of 2D gel electrophoresis (Chen et al., 2005).

We detected PAI-1 in media conditioned by fetal and young pig S-V cell cultures, and PAI-1 gene expression was evident in adipose tissue and S-V culture microarrays. These are the first reports of PAI-1 secretion by fetal S-V cultures and PAI-1 gene expression by fetal S-V cultures and fetal adipose tissue. Conditioned media PAI-1 levels were not influenced by hormone-induced differentiation as observed in studies of human preadipocytes (Crandall et al., 1999; 2000a). Our studies also show that PAI-1 levels or secretion were not associated with fetal age-associated adipocyte differentiation. Regardless, the endogenous expression and secretion of PAI-1 may facilitate preadipocyte migration and clustering (Crandall et al., 2000b). Nevertheless, these studies indicate that adipose tissue S-V cells could represent a major source of circulating PAI-1 in the pig.

Our collective studies indicate that APO-A1 is a major component of media conditioned by porcine S-V cell cultures. The presence of APO-A1 in fibroblast-conditioned media was largely attributed to previous exposure to fetal bovine serum (Lim and Bodnar, 2002). However, we demonstrated, herein, detectable APO-A1 mRNA in S-V culture microarrays and APO-A1 levels in conditioned media that were independent of FBS exposure. For instance, APO-A1 levels in fetal S-V cell cultures increased with time in culture, adipocyte differentiation, and age of S-V cell donor. Furthermore, an APO-A1-like string of proteins (Sanchez et al., 1995; Lim and Bodnar, 2002; Leak et al., 2004) was clearly detectable in completely serum-free S-V cell cultures. We also demonstrated that APO-A1 was a major protein in developing fetal adipose tissue and detected APO-A1 mRNA in neonatal adipose tissue microarrays. Immunocytochemistry of fetal adipose tissue showed that APO-A1 was only present in fetal adipocytes (G. J. Hausman, unpublished observations). Furthermore, APO-A1 protein was also detected in human and mouse adipose tissue in several proteomic studies (Lanne et al., 2001; Sanchez et al., 2001; Corton et al., 2004). There are no reports or publications on the influence of APO-A1 on adipogenesis. Therefore, the present studies are the first to show a potential relationship between APO-A1 and adipogenesis *in vitro*. However, our *in vivo* studies show no such relationship because APO-A1 protein levels in fetal adipose tissue were similar before and after the onset of adipogenesis. Significant levels of PPAR γ (Kim et al., 2000) and C/EBP α (Lee et al., 1998) proteins were also detected in 50-d fetal pig adipose tissue (i.e., before the onset of adipogenesis). Consideration of other cell types in immature adipose tissue is relevant to potential sources of APO-A1 and other proteins. For instance, electron microscopic studies of 50-d and older fetal adipose tissue demonstrated a wide variety of cell types including macrophages associated with necrotic cells and high cell turnover (Hausman and Richardson, 1982). Furthermore, capillary endothelium (in the absence of adipocytes) in 70-d fetal adipose tissue phagocytosed lipid droplets after liposyn injection and displayed other macrophage ultrastructural traits (G.

J. Hausman and L. R. Richardson, unpublished observations). The extracellular matrix of vascular endothelial cells is differentiated before the onset of adipocyte differentiation in fetal adipose tissue (Hausman et al., 1991). Adipocytes, endothelial cells, and macrophages may have overlapping functions and share a common lineage because preadipocyte and macrophage phenotypes are very similar and preadipocytes can convert to macrophages (Charriere et al., 2003). In this regard, macrophages express both the C/EBP α and PPAR γ genes (Sun et al., 2003). Several studies indicate that adipocytes and endothelial cells may also have a common progenitor (Wright and Hausman, 1990; Planat-Benard et al., 2004). Porcine endothelium expresses the APO-A1 gene *in vivo* (Mockel et al., 1994), and APO-A1 is bound to the macrophage plasma membrane (Gaus et al., 2004). Therefore, in developing adipose tissue nonadipocyte cell types may represent a major source of secreted factors.

It is important to note that some of the apparently unexpected or unusual results of the current study are, in fact, not unusual based on other studies. For instance, a high proportion of nonpig protein hits was reported in the current study and in a proteomic study of pig brain tissue in which 90% of total protein hits were nonpig hits and represented 5 species (Friso and Wikstrom, 1999). Furthermore, the expression of genes or secretion of proteins considered unusual or novel for adipose tissue were reported in the present and other studies. For instance, secreted factors including the FSH β subunit, LH β subunit, and corticotrophin-releasing hormone were also detected in human adipose tissue microarray studies (Yang et al., 2003a,b; Gomez-Ambrosi et al., 2004). Furthermore, a number of additional secreted factors including IL-2, IL-4, IL-5, IL-7, IL-18, IFN β , IGFBP-1, APO-A1, APO-A4 were detected in a mouse adipose tissue microarray study (Ross et al., 2002). Proteins typically considered nonsecreted such as cytoskeletal proteins and enzymes were detected in media conditioned by S-V cell cultures (present study) and in media conditioned by fibroblasts and adipocytes (Chen et al., 2005; Lim and Bodnar, 2002; Prowse et al., 2005). Examples of nonsecreted proteins include, cyclophilin A, B (rotamase), beta actin, fatty acid synthase, malate dehydrogenase, vimentin, serine/threonine protein phosphatase, and phosphomevalonate kinase (Table 5; Lim and Bodnar, 2002; Chen et al., 2005; Prowse et al., 2005). In particular, hemoglobin beta chain protein was detected in S-V conditioned media and in media conditioned by rat adipocytes (Chen et al., 2005). We also detected hemoglobin beta chain protein in fetal S-V culture cell homogenates (data not shown). Hemoglobin gene expression has been reported in rodent and human adipose tissue by microarray (Gabrielsson et al., 2000; Nadler et al., 2000; Castro-Chavez et al., 2003; Castro-Chavez, 2004) and differential display analysis (Lopez et al., 2004). Therefore, media conditioned by S-V cell cultures share several unusual or

unexpected characteristics of media conditioned by adipocytes and fibroblasts.

The current study and others demonstrate that adipose tissue expresses genes for a number of growth factors and related proteins that may influence adipogenesis in a paracrine or autocrine manner. For instance, expression of acidic FGF (FGF1), FGF-7, NGFB, and HBEGF, herein, is consistent with FGF (FGF1), FGF-7, and HBEGF expression in human adipose tissue microarrays (Gabrielsson et al., 2002; Matsuzawa et al., 2003) and NGFB expression by mouse adipose tissue (Peeraully et al., 2004). A role for FGF1 in human adipogenesis was recently demonstrated in vitro (Hutley et al., 2004). Heparin binding EGF downregulates IGFBP (Provenzano et al., 2005) and mediates IGF-I receptor-driven mitosis (Mulligan et al., 2002). As expected, IGF-I, -II, IGFBP-2, -3, -5, and -6 genes were detected in the current study and in many microarray studies of rat, mouse, and human adipose tissue (Nadler et al., 2000; Ross et al., 2002; Ruan et al., 2002; Lopez et al., 2003; Gomez-Ambrosi et al., 2004; Higami et al., 2004; Urs et al., 2004; Vohl et al., 2004). The IGF system proteins mediate hormone-driven adipogenesis (review, Hausman et al., 2001).

Adipose tissue and S-V cells express many secreted angiogenic factors (reviewed, Hausman and Richardson, 2004) including ANGPT-2, TIMP-1, -2, and VEGF reported in the current study. Secretion of these and other angiogenic factors and angiogenesis would undoubtedly accompany adipogenesis in neonatal adipose tissue.

The current evidence indicates that of all the adipose tissue secreted factors only IL-6, IL-8, PAI-1, leptin, and adiponectin can be considered true endocrine factors (review, Hauner, 2005). Furthermore, when administered centrally, IL-1 β , IL-6, IL-8, and TNF α decrease food intake in rodents (Plata-Salaman and Borkoski, 1993; Plata-Salaman et al., 1996; Wallenius et al., 2002). Therefore, the current study demonstrates that pig adipose tissue secretes several endocrine factors, which could affect growth and performance. Further research is necessary to examine adipose tissue secretion of endocrine factors throughout growth and development. Regardless, the current studies greatly expand the list of pig adipose tissue-secreted factors.

Impact

There has been a lack of information on factors secreted by adipose tissue from meat animals compared with other species. Fetal and neonatal porcine adipose tissue expresses many typical adipose tissue-secreted factors, many of which influence fat cells and fat cell development at the local level. This work indicates a highly complex and extensive group of locally acting factors secreted by porcine adipose tissue and provides new possibilities to study local regulation of porcine fat cell development. Fetal and neonatal porcine adipose tissue also secretes several factors capable of influenc-

ing central metabolism and growth. Therefore, it will be important to examine these and other adipose tissue factors during growth and pubertal development in the pig.

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